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Method for the Quantitative Morphologic Analysis of Tissues

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A perennial technical problem in histology and pathology is the difficulty of obtaining quantitative data on the spatial distribution of morphologic tissue components and their changes in growth, functional activity, and disease. It is hoped that the method here presented will prove of use in this respect.

The requirements for such a method are (1) that it be sufficiently accurate, (2) that it be applicable to tissues of considerable complexity, (3) that it be simple, and (4) that it be possible to subject it to independent check by other methods when necessary. The method proposed fulfills these requirements within reasonable limits. Heretofore, the method mainly depended on has been the transfer of the outlines of the microscopic image of the tissue component of a section, as seen on the slide, to paper either by means of a camera lucida or by photography, with subsequent measurement of the components of interest, such as cells, nuclei, etc., by use of the planimeter or by the excision and weighing of the outlined areas. This method is at present commonly limited to the measurement of cells and nuclei and is not used for even semi-routine procedure by reason of the inordinate amount of time required. What is ordinarily required from such measurement is to secure, not the absolute area or volume of the components measured, but the ratio between the volumes or areas, particularly the nucleocytoplasmic ratios, or the volume ratios of active and inactive gland cells. The proposed method directly ascertains such ratios and obviates the necessity for tedious drawing and measurement.

METHOD

Let us consider a slice of tissue of relatively homogeneous structure such as a section of liver; let us also conceive of a mathematical point moving through that tissue and following a random path in three dimensions. It is obvious that the proportion of its path resting in the nuclei, in the cytoplasm, in the vascular spaces, or bile ducts will, if the path is indefinitely prolonged, approach as a limit the proportions of the fractions of volume occupied by these morphologic constituents.

To make the measurements of the path as described is, of course, impossible, but the problem can be approached quite simply in another way. By substituting for the continuously moving point a random distribution of points throughout the tissue and summing up the number of points lying in the various components, the ratios of these sums will also approach the ratios of the volume fractions occupied by the respective components. The shape of the individual component units does not enter into this consideration.

If a method of ascertaining the sums of points as envisaged can be devised, the ratios between such sums can be ascertained for the components of a given unit area or volume; and if additional measures are obtained in respect to the components to give the number of units of each component in a given area or volume, the area or volume of each component unit can be

readily obtained. Hence, by using these additional measures it should be possible to check the results obtained with such a method against those acquired with the usual methods, which start by ascertaining the volumes or areas of the component unit and calculating the ratios. A description of the means of applying this concept and testing it against the usual methods follows.

The fundamental requirement is to obtain a random distribution of points within the tissue under observation. This can be done either by random scattering of points through the tissue, or random presentation of the tissue at a fixed point or a simple pattern of points. The latter procedure is the more practical for the purpose.

All that is required is to provide a means for indicating a point or points in the focal plane of a microscope image and for obtaining random movement in all dimensions of the specimen to be observed. The ordinary mechanical stage plus the focusing range of the microscope is sufficient for the latter, and the point or pattern may be obtained by cementing a hair or several hairs to the diaphragm of the ocular to act as finders. The hairs then appear in the focal plane of the image, and their free extremities constitute the point pattern. In practice the object is brought into focus, the position of the points noted, i. e., whether they fall on nucleus, cytoplasm, tissue space, etc., and these observations are recorded. The object is then thrown completely out of focus and by means of the mechanical stage is moved under the objective. It is then brought back into focus at varying vertical levels, and the position of the points is again noted and recorded. This process is repeated, following a fairly systematic pattern in all three dimensions and covering a sufficient portion of the tissue measured to give an adequate sample. It is not necessary to avoid the accidental repetition of measurement of the same space. Point-for-point repetition (unless deliberate) is almost impossible.

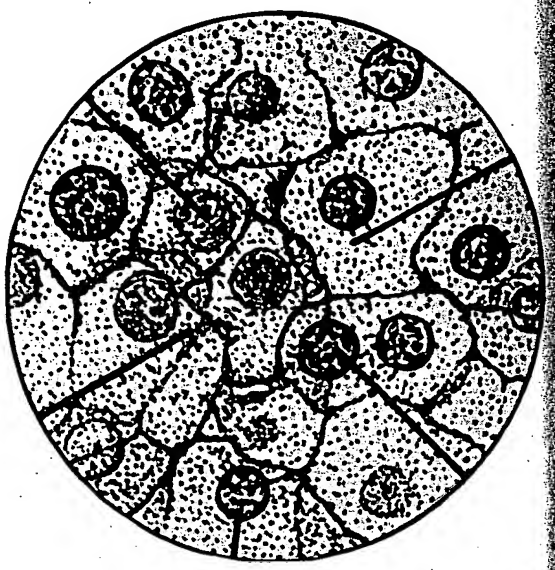

FIGURE 1.—Point pattern as seen in making measurements. The upper point is focused on a nucleus to serve as reference. The upper left point coincides in focus on a nucleus, the lower right one is over a nucleus, but the nucleus is not in focus. The other points are on cytoplasm. The reading is one hit on nucleus, three on cytoplasm.

In actual practice, a purely psychologic but very real difficulty arose. A trained observer, when using a finder such as the glued hairs, is accustomed to focusing in such a way that the finder coincides with some object. It was very difficult not to do this and thus bias the observations, especially when dealing with thick sections. To avoid this difficulty, a very short extra hair is glued on the eyepiece diaphragm; then the focus is so made that the tip of this hair is brought into coincidence of focus with a selected object, usually a nucleus. The position of this point is not recorded, but the other points alone are used. It is best to keep this short point at the upper limit of the field, since by so doing, the attention is concentrated there-

on and the positions of the other points are not noticed. As there is in this type of observation no significant relation between the position of this point and the others used, the positions of the points used are truly attained at random. The arrangement is shown in figure 1. As will be shown, this allows one to deal easily with thick or thin sections without introducing errors. (A method for the empirical determination of the number of observations necessary to constitute an adequate sample will be described later.) The "hits"[1] on each element are summed and the ratios calculated. The tests described in the following section show that satisfactory results can be obtained with this simple procedure.

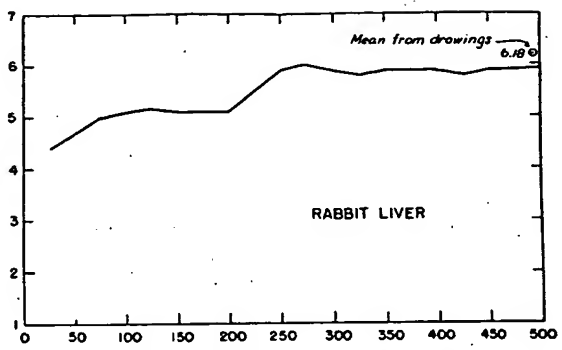

FIGURE 2.—March of the cumulative mean of the ratio of hits on nucleus to hits on cytoplasm of parenchymal cells on a section of rabbit liver, with increasing numbers of observations. Ordinate, ratio; abscissa, number of hits on nuclei. Note that the mean is stable with a total of 350 or more hits on nuclei. The ratio of average area of nucleus to cytoplasm as calculated from measurements of 50 camera lucida drawings agrees well with the ratio found.

TESTS

The first test of the method was made on a section of rabbit liver. The tissue preparation had been stained in such a manner as to show the cell outlines clearly; and this characteristic, since it enabled one to make camera lucida drawings, was used in checking the results. Observations were made by using a 20+ ocular and a 2-mm. oil-immersion objective, and the hits recorded on liver nuclei, liver cytoplasm, and all other components, these last being designated collectively "tissue space." The ratio of hits on cytoplasm to hits on nuclei was 5.82 (σ=1.22).[2] To check this result, camera lucida drawings were made from the same preparation of the outlines of 50 individual cells and their nuclei, selected for the ease with which the drawings could be made. The areas of all were measured with a planimeter, and the ratio between the average area of the nucleus and the cytoplasm was calculated. The value obtained was 6.2, σ1.52, a difference of 6.9 percent from the lower value. This seemed a satisfactory agreement considering the small number of cells measured.

In securing the data by the proposed method a sufficient number of observations was made to secure 500 hits on nuclei. To ascertain whether this number constituted a sufficient sample, the data were plotted to show the ratio as calculated from the successive totals of hits on nuclei and cytoplasm, as successive groups of 25 hits on nuclear material were obtained. A similar cumulative plot was made of the ratio for each successive group of cells and nuclei drawn and measured (fig. 2). The ratios thus plotted show less and less variation as the number of measurements accumulate, and it is fairly easy to judge to what extent they are likely to vary and thus to obtain an empirical estimate of the size of sample necessary for a reliable measurement.

If all measurements are so plotted and it

[1] *A "hit" is defined as the contact of the image of the point with the clearly observed image of the specimen.*

[2] *In all instances where the standard deviation is given for observations by this method, the deviations are those of a unit observation consisting of an arbitrarily determined group of hits, usually the number of hits per 25 hits on the element used as standard, in this case the nucleus.*

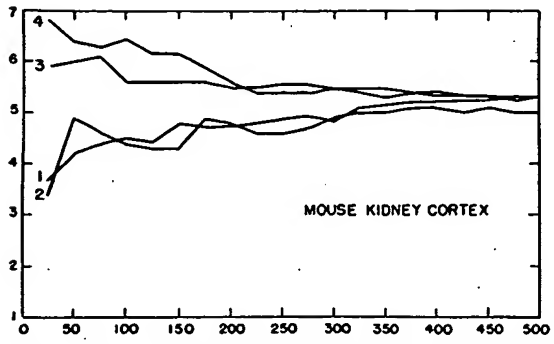

FIGURE 3.—March of the mean ratio of hits on nuclei to hits on cytoplasm of the tubule cells of a section of mouse kidney cortex for 4 sets of observations, each including 500 hits on the nuclei. Note the centering and stabilizing of the ratios with increasing number of observations, giving reproducible results.

is thus ascertained before applying statistical tests that a stable mean has been obtained, the effort of applying elaborate tests to intrinsically insufficient data is avoided.

In the next test of the method, that for reproducibility, a section of mouse kidney cortex was used. Bouin's fixation, paraffin embedding, and haematoyxlin-eosin stain were employed. Measurements were made of the tubule cells, and hits on nucleus, cytoplasm, and tissue space recorded. As before, all material other than tubal was designated tissue space. Four runs were made, and cumulative plots of the ratios made for each run (fig. 3). This shows that the method has a high degree of reproducibility on given material, the final nucleocytoplasmic ratios being 5.0, $\sigma=1.30$; 5.3, $\sigma=1.02$; 5.3, $\sigma=0.95$; 5.3, $\sigma=0.8$ for the respective runs, or an average spread of 4.4 percent of the lowest value obtained.

A further empirical test was made, that of the ability to deal with a section of a complex organ. A section of the mouse kidney was again used, and a record was kept of hits made on tubule cell nuclei, tubule cell cytoplasm, tubule lumina, glomeruli, and vascular space. The examination was confined to the cortex. The number of hits on the glomeruli was used as the index and the recording was carried on until 700 hits had been scored on glomerular tissue. The ratios were computed as between tubule cytoplasm and nucleus; tubule cells and tubule lumina plus glomeruli plus vessels; tubule cells and lumina; and tubule cells and glomerular tissue. The next step was to calculate the average areas of certain tissue elements, tubule cells, tubule lumen, and glomerulus. For the calculation it was necessary to have the following further measurements all at the magnification used: (1) The area of the tubule nucleus; (2) the area of the microscope field; (3) the average number of lumina per field; and (4) the average part of a glomerulus per field. The first two requirements were satified by making (1) camera lucida drawings of 600 nuclei, and (2) a similar drawing of the outline of the field, and measuring them with a planimeter. The last two requirements were met by counting the lumina and the glomeruli seen in a number of fields and dividing the total number of each by the number of fields counted. The results are given in table 1. It is obvious that all counts and drawings must be at the same magnification; or allowance should be

TABLE 1.—*Values obtained in analysis of mouse kidney cortex*

Description	Value	Standard deviation (σ)	Symbol
Area of microscope field	3,419		*A.*
Area of tubule nucleus	15.4± 0.12	2.92	*N.*
Ratio, Hits on tubule cells/Hits on other elements	5.51± .18	.94	*C.*
Ratio, Hits on tubule cytoplasm/Hits on tubule nuclei	5.38± .15	.83	*n.*
Ratio, Hits on tubule cells/Hits on tubule lumina	14.20± .87	4.60	*L.*
Ratio, Hits on tubule cells/Hits of glomeruli	15.70± .54	2.89	*G.*
Lumina per field...number	1.31± .034	.136	*l.*
Gomerulus per field..percent	5.38± .15	.83	*g.*

made for difference, if changes in this respect are made for reasons of expediency.

From the values given in table 1 it is possible to calculate (1) the average area of the tubule cell, (2) the average area of the tubule lumen, and (3) the average area of a glomerulus. They are as follows:

(1) $N(n+1)=$ the average area of the tubule cell,

(2) $A-\left(\frac{A}{1+C}\right)=$ the average area in tubules per field,

then $\frac{A-\left(\frac{A}{1+C}\right)}{L}=$ average area of luminal space,

and $\frac{\frac{A-\left(\frac{A}{1+C}\right)}{L}}{l}=$ average area of lumen.

(3) $\frac{A-\left(\frac{A}{1+C}\right)}{G}=$ average glomerular area per field,

then $\frac{\frac{A-\left(\frac{A}{1+C}\right)}{G}}{g}\times 100=$ average area of glomerulus.

These computations give predictions of the average areas of tubule cell, tubule lumen, and glomerulus. These values were checked independently by making camera lucida drawings of the outline of cells, lumina, and glomeruli, measuring them with a planimeter, and calculating their average areas. The results are given in table 2. The differences are all less than 5 percent of the lower values. This is a satisfactory agreement.

TABLE 2.—*Comparison of calculated and directly measured values for different elements of tissue section*

Element	Average area		Difference
	Calculated value	Measured value	
Tubule cell	98.2	[1] 99.5± 2.5	1.3
Tubule lumen	155.5	160.8±17.6	5.3
Glomerulus	1,644.6	1,602.8±44	41.8

[1] Large errors are due to the fact that comparatively small numbers were used in check measurements. The means were accepted as soon as they appeared reasonably stable in cumulative plot.

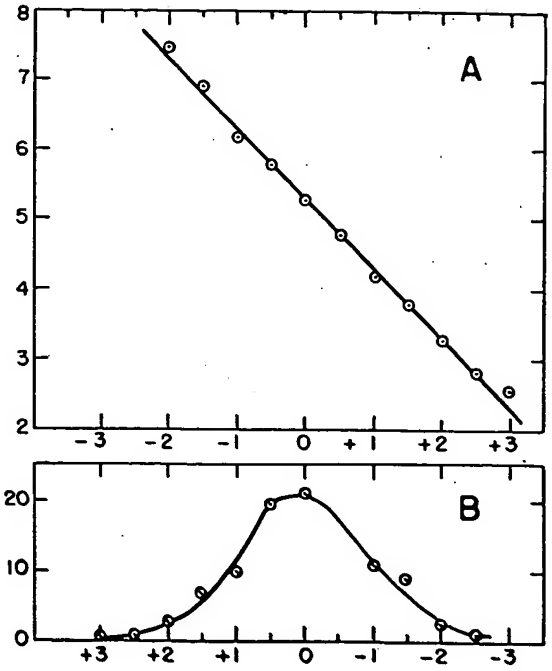

FIGURE 4.—Two plots of the distribution of the deviations about their means, of all unit observations in terms of deviation units. *A*, Probit curve; *B*, usual frequency distribution curve. Ordinate: *A*, probits; and *B*, number of deviations. Abscissa: both *A* and *B*, departure from mean values, positive and negative, in standard deviation units.

Two more tests remained to be made. The theory presumes a normal distribution of the variates. All variates were converted into standard deviation units and plotted. The distribution curve is shown in figure 4, *B*. An analyses was also made by the method of Bliss [3] to see if the distribution follows a normal curve. The integrated percentage frequencies were transformed to probability units (probits) and plotted against deviation in standard deviation

[3] *Bliss, C. I.: The comparison of dosage-mortality data. Ann. Appl. Biol., 22: 307–333 (1935).*

TABLE 3.—*Variation in nucleocytoplasmic ratio as calculated from measurements made on sections of differing thickness cut from the same block of mouse liver*

Thickness of section (in microns)	Ratio Cytoplasm / Nucleus	Difference
1		
3	10.10	0.04
5	10.56	.42
10	10.01	.13
15	9.90	.24
Mean	10.14	[1] .21

[1] Less than 3 percent of the mean.

units. The excellent fit to the straight line obtained by this transformation is shown in figure 4, *A*, and indicates that the frequency distribution of the variates does follow a normal curve. Since the hits are distributed in three dimensions, the measurements and therefore the ratio between two tissue components should be practically independent of the thickness of section used. This second test was made by measuring the hits on cytoplasm and nucleus in a series of sections of differing thickness cut from a single block of mouse liver. The results are given in table 3. The standard deviation is less than 3 percent of the mean, and the differences between the ratios found are evidently not related to the thickness of the sections.

ANALYSIS OF ORGANS

The method can readily be applied to the analysis of the whole or part of an organ. It has been applied with good results to the mouse anterior pituitary to ascertain the composition in terms of the morphologic components. It is thought that a sample of such an analysis may be of value, therefore the data for a single gland are presented herewith.

The material, furnished by Dr. Dalton, of this Institute, consisted of the complete serial sections of the anterior pituitary of a young adult male mouse of the C3H strain. Measurements were made on every ninth section, 8 sections in all; 2 sets of measurements were made on each section, each set being based on 25 hits on α nuclei per section. This was done to secure an adequate sample from the whole organ, since tests showed that an estimate based on a single section was totally inadequate. Hits were recorded for α cell cytoplasm, α cell nucleus, β cell cytoplasm, and β cell nucleus.

The cumulative plots of the means as percent of total hits are shown in figure 5. The greatest difference between any 2 sets of measurements on 1 component is less than 1 percent. This difference is exceptionally low, but from the data at hand (some 25 or more such measurements) the

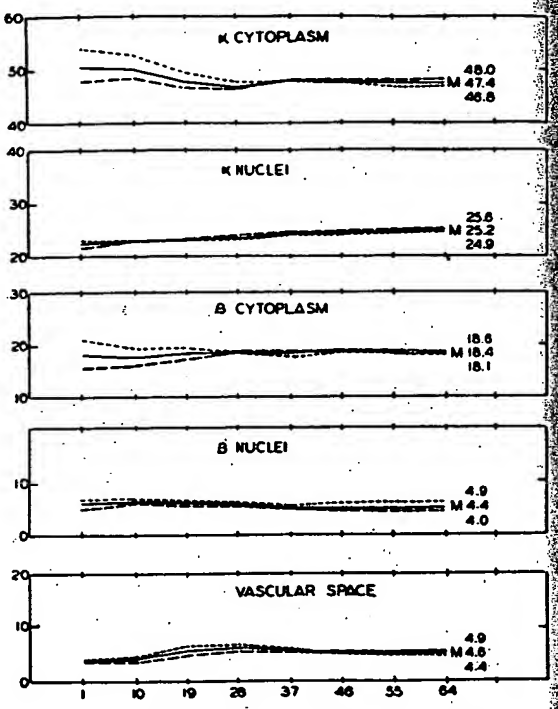

FIGURE 5.—Plots of the collective means of percentage of α cytoplasm, α nucleus, β cytoplasm, β nucleus, and vascular space for analysis of anterior lobe of a mouse pituitary. Dotted lines, march of means of groups of hits, giving 25 hits on α nucleus per section. Solid line, march of means of the 2 groups per section. Ordinates, percent of total hits scored. Ordinate scale, section number from serial sections cut at 3μ.

average standard deviation between such pairs of measurements is 1.8 percent. One can expect a standard deviation of definitely less than 4 percent. The time required to get such a series of measurements on 1 gland is less than 2 days. The data allow the calculation of several ratios, as between nucleus and cytoplasm, or cell and cell type, which would require a much longer time to obtain by any other method known to the writer.

DISCUSSION

It is necessary to emphasize that while the method permits calculation of such ratios as α cell to β cell, it does not give information about the ratio between the number of α cells and the number of β cells. The ratio obtained is that of the fractions of unit volume occupied by β cells to the fraction of unit volume occupied by α cells as determined by hits on β and α cells, respectively.

Most of the data of this sort in the literature deal with ratios between numbers of cells. In making comparisons between organs, the assumption is that all cells of a given type are alike in the organs compared. This the writer believes is probably untrue. The assumption itself is certainly fallacious.

In the method presented herein, no assumption as to number of cells or other components enters into consideration, the comparison being made between two or several fractions of volume, or of fractions of volume to total volume. This procedure appears to be preferable since it would seem a priori more likely that the physiologic, physical, or chemical significance of a component is directly linked to its proportional volume in a given organ or organism than to the number of compartments into which that volume is divided. If the latter information is needed, it can be readily obtained.

As in all experiments where a method of random sampling is used, care must be taken to design all experiments so that adequate samples are obtained.

SUMMARY

A method is described for measuring directly the volumetric or area ratios of components of tissues or organs under any conditions, where the various components are clearly distinguishable visually.

Tests of the method showing its degree of accuracy when applied to the analysis of fixed and stained preparations are presented.